# Hypolipidemic Effects and Absorption of Citrus Polymethoxylated Flavones in Hamsters with Diet-Induced Hypercholesterolemia

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Formulations containing citrus polymethoxylated flavones (PMFs), mainly tangeretin, or citrus flavanone glucosides, hesperidin and naringin, were evaluated for cholesterol-lowering potential in hamsters with diet-induced hypercholesterolemia. PMF metabolites were also investigated. Diets containing 1% PMFs significantly reduced serum total and very low-density lipoprotein (VLDL) + LDL cholesterol (by 19−27 and 32−40%, respectively) and either reduced or tended to reduce serum triacylglycerols. Comparable reductions were achieved by feeding a 3% mixture of hesperidin and naringin (1:1, w/w), implying lower hypolipidemic potency of the hesperidin/naringin vs PMFs. HPLC-MS analysis identified high serum, liver, and urine concentrations of tangeretin metabolites including dihydroxytrimethoxy-flavone and monohydroxytetramethoxyflavone glucuronides and aglycones. Total liver concentrations of tangeretin derivatives corresponded to hypolipidemic concentrations of intact tangeretin in earlier experiments in vitro. This suggests that PMFs are novel flavonoids with cholesterol- and triacylglycerol-lowering potential and that elevated levels of PMF metabolites in the liver might be directly responsible for their hypolipidemic effects in vivo.

**KEYWORDS: Polymethoxylated flavones; cholesterol; triacylglycerols; hamster; tangeretin metabolites; serum; liver; urine**

## INTRODUCTION

Citrus juices and their constituent flavonoids have been shown to produce a number of health benefits, including anticancer and antiinflammatory effects (reviewed in *1*). Several reports also suggest that high dietary intake of orange juice or grapefruit juice might reduce hypercholesterolemia (*2*, *3*), and this was postulated to be largely due to the principal citrus flavanones, hesperetin from oranges and naringenin from grapefruit, both found as their glycosides, Hesp and Nar. Absorption and bioavailability studies showed that Hesp and Nar are bioavailable after deglycosylation by intestinal bacteria or by endogenous glycosidases (*4*). In humans administered citrus juices or pure citrus flavanone glycosides, the combined urinary recoveries of metabolites, largely glucuronides, ranged between 1 and 25%, depending on the ingested dose, type of formulation, and individual responses (*5*−*7*).

The hypolipidemic effects of hesperetin and naringenin have been investigated in human hepatoma HepG2 cells, the common in vitro model used to study the regulation of hepatic production and catabolism of atherogenic, apolipoprotein B (apo B)-containing lipoproteins such as VLDL and LDL (*8*). Exposure of HepG2 cells to hesperetin and naringenin dose dependently reduced the net secretion of LDL-associated apo B, and the apo B $IC_{50}$ concentrations (concentrations required to reduce medium apo B by 50%) were determined to be 142 and 178 $\mu$M for hesperetin and naringenin, respectively (*9*, *10*). The mechanism of action of hesperetin and naringenin in cells has been since examined in detail (*11*, *12*). The hypolipidemic potential of Hesp and Nar was also studied in vivo. Dietary supplementation with 0.5% of Hesp or Hesp/Nar mixtures lowered serum cholesterol levels in rats and in ovariectomized mice (*13*, *14*), but the similar effect was not produced in rabbits (*15*).

In addition to the flavanone glycosides such as Hesp and Nar, flavonoid compounds in citrus include highly methoxylated flavones, termed PMFs. The most common citrus PMFs, Tan and Nob (**Figure 1**), occur in orange, tangerine, and sour orange peel (*Citrus aurantum*) (*16*). While previous studies demonstrated that PMFs exhibit anticancer and antiinflammatory actions in cells and in animals (reviewed in *1*), their possible roles in the modulation of lipid metabolism have not been investigated. Recently, we showed that several natural and synthetic PMFs possess the ability to inhibit the net secretion of apo B in HepG2 cells. Among the compounds studied, Tan and Nob were the most active, reducing medium apo B by 81−86% when added to cells at a concentration of 67 $\mu$M for the

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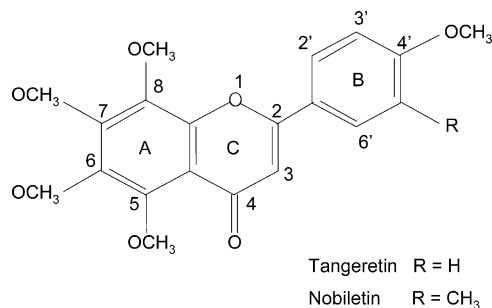† KGK Synergize Inc..
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Tangeretin   R = H
Nobiletin   R = CH₃

**Figure 1.** Structure of Tan and Nob.

period of 24 h. The apo B $IC_{50}$ values of Tan and Nob (6.7 and 13.2 $\mu$M, respectively) were much lower than those for hesperetin and naringenin (*17*). Thus, we hypothesized that PMFs might exhibit greater hypolipidemic potential in vivo than Hesp and Nar, providing that their absorption is comparable to that reported for citrus flavanones.

The absorption and biotransformation of PMFs have been recently investigated in selected in vivo and in vitro systems. Urine collected from rats administered Tan at 100 mg/kg body weight/day for 14 days contained a number of glucuronic acid and sulfate Tan derivatives but no intact free flavone. All Tan products found in the urine were identified as monohydroxy-tetramethoxy-, dihydroxytrimethoxy-, or dihydroxytetramethoxy-flavone conjugates, and the cumulative excretion of these compounds amounted to approximately 11% of the administered daily dose (*18*). Another experiment showed that rats given a single oral dose of Nob excreted five different Nob metabolites including three monodemethylated isomers (*19*). The formation of partly hydroxylated PMF derivatives was also observed in vitro, following the exposure of rat, mouse, or human liver microsomes to Tan (*20, 21*) and following the incubation of rat liver microsomes or human intestinal Caco-2 cells with Nob (*22*). The above data, although limited, suggested that PMFs are bioavailable, and their metabolites, if as biologically active as intact PMFs, might reach concentrations in the liver tissue sufficient to modulate serum and liver lipid metabolism.

Our present study was therefore undertaken to investigate the effects of three dietary PMF supplements, Tan, a standardized Tan/Nob mixture, and a commercial extract containing PMFs from tangerines, on lipid metabolism in hamsters with casein-induced hypercholesterolemia. The hypolipidemic responses induced by 1.0% PMFs were also compared to those produced by 3.0% supplement containing Hesp and Nar in a 1:1 proportion. The third important objective was to identify PMF metabolites in serum, liver, and urine from hamsters fed Tan- and Tan/Nob-supplemented diets and to estimate concentrations of these compounds in tissues and urine.

## MATERIALS AND METHODS

**Materials.** Tan (97.4% pure) and a Tan/Nob formulation containing 93% PMFs (63% Tan, 26% Nob, and 4% other minor PMFs including primarily 5-desmethylnobiletin) were isolated from winterized tangerine peel oil precipitate. Tangerine oil precipitate was collected and washed extensively with hexane to remove the volatile oil constituents, mainly limonene, and other hexane soluble contaminants. The hexane-washed residue was dissolved in chloroform, filtered, dried by rotary evaporation, and analyzed for PMF content. The purified Tan was obtained as previously described (*23*). The commercial 48.1% PMFs extract containing 22.0% Nob, 16.0% sinensetin, 2.4% Tan, and 7.7% of unidentified PMFs from tangerines was obtained from Food Ingredient Technologies, Australia PTY Ltd. Hesp and Nar were purchased from Sigma (St. Louis, MO).

**Table 1.** Percent Composition of Experimental Diets[a]

| ingredient | control | 0.25% Tan/Nob | 1.0% Tan, 1.0% Tan/Nob, or 1% PMF[T] | 3.0% Hesp/Nar |
|---|---|---|---|---|
| casein | 26.3 | 26.3 | 26.3 | 26.3 |
| rice flour | 39.8 | 39.6 | 38.8 | 36.8 |
| coconut oil | 10 | 10 | 10 | 10 |
| safflower oil | 2 | 2 | 2 | 2 |
| cellulose | 7.5 | 7.5 | 7.5 | 7.5 |
| wheat bran | 7.5 | 7.5 | 7.5 | 7.5 |
| choline chloride | 0.3 | 0.3 | 0.3 | 0.3 |
| potassium bicarbonate | 2 | 2 | 2 | 2 |
| vitamin mix | 1 | 1 | 1 | 1 |
| mineral mix | 3.5 | 3.5 | 3.5 | 3.5 |
| cholesterol | 0.1 | 0.1 | 0.1 | 0.1 |
| PMF supplements | | 0.25 | 1 | |
| Hesp/Nar supplement | | | | 3 |

[a] Casein (high nitrogen), rice flour, choline chloride, and potassium bicarbonate were obtained from ICN Biochemicals. Wheat bran was purchased from Bio-Serv (Frenchtown, NY). Vitamin mix (no. 4006), mineral mix (no. 170910), and other basic ingredients are from Harland-Teklad (Madison, WI).

**Animals and Diets.** The animal protocol was in accordance with the Canadian Council of Animal Care guidelines and was approved by the University of Western Ontario Council on Animal Care. Male Golden Syrian hamsters, weighing 100−120 g, were housed individually in a constant temperature of 21−24 °C and with a 12 h light:dark cycle and fed ground chow diet for 1 week. After that, they were divided into groups of 5−10 animals with similar average body weights and fed experimental diets for 35 days. The composition of the powdered basal diets was described by Potter et al. (*24*). All diets contained 25% casein, with or without supplements of Tan, Tan/Nob formulation, mixed PMF[T], or Hesp + Nar, 1:1, w/w (Hesp/Nar) at indicated levels (**Table 1**). Food and water were provided ad libitum. Body weights were monitored weekly, and food consumption was estimated during the last 2 weeks of treatment for three consecutive days.

Two experiments were conducted to address study objectives. In experiment 1, effects of pure Tan and a Tan/Nob formulation on serum and liver lipids were examined by feeding diets containing 1.0% Tan, 0.25% Tan/Nob, and 1.0% Tan/Nob. In addition, liver and serum samples and aliquots of urine collected during the last week of treatment were saved for identification and measurement of PMF metabolic products, as described below. In experiment 2, another formulation containing 1.0% PMF[T] was tested for its ability to modulate serum and liver lipids and its effects were compared to those obtained for 3.0% Hesp/Nar.

At the end of each study, fasting blood samples were collected and serum total and HDL cholesterol as well as serum triacylglycerols were measured by the enzymatic timed end point method, using Beckman Coulter reagents (CHOL reagent, HDLD HDL reagent, and TG triglycerides GPO reagent, respectively). All measurements were performed using an automated SYNCHRON LX system. VLDL + LDL cholesterol were calculated as a difference between total and HDL cholesterol. Liver samples were collected for determination of choles-terol and triacylglycerols using a published enzymatic method (*25*). The statistical analysis was done by one way analysis of variance (ANOVA) followed by Dunnet's test or by Student's *t*-test.

**Analysis of PMFs Metabolites.** Urine, liver, and serum samples were freeze-dried and extracted with dimethyl sulfoxide overnight with continuous shaking. HPLC separations of extracts were achieved using a Nova-Pak C18 column (15 cm × 3.9 mm i.d.) (Waters Inc., Milford, MA). Elution conditions included a three solvent gradient composed initially of water/acetonitrile/2% formic acid (85/10/5, v/v/v) and increased with linear gradients to 55/40/5, v/v/v over 15 min, then to 50/45/5, v/v/v at 45 min, and to 5/90/5, v/v/v at 55 min with a flow rate of 0.75 mL/min. Instrumentation included a Waters 2695 Alliance HPLC coupled with Waters 996 PDA and Waters ZQ single quadrupole mass detectors. PDA detection was monitored between 400 and 230 nm. Data handling was done with MassLynx software ver 3.5 (Micromass, Division of Waters Corp., Beverley, MA). HPLC condi-

Citrus Polymethoxylated Flavones in Hamsters

*J. Agric. Food Chem.,* Vol. 52, No. 10, 2004  **2881**

**Table 2.** Growth Performance of Hamsters in Experiments 1 and 2

| diet | initial weight (g) | growth rate (g/day) | food consumption (g/day) | food efficiency |
|---|---|---|---|---|
| | | experiment 1 | | |
| control | 119.2 ± 5.4 | 0.60 ± 0.22 | 7.6 ± 0.8 | 0.09 ± 0.02 |
| 0.25% Tan/Nob | 119.5 ± 7.8 | 0.55 ± 0.24 | 7.0 ± 0.7 | 0.07 ± 0.02 |
| 1.0% Tan/Nob | 119.1 ± 9.1 | 0.47 ± 0.12 | 6.1 ± 1.6 | 0.08 ± 0.02 |
| 1.0% Tan | 118.9 ± 7.9 | 0.52 ± 0.13 | 7.4 ± 1.4 | 0.08 ± 0.02 |
| | | experiment 2 | | |
| control | 119.8 ± 10.2 | 0.62 ± 0.24 | 8.8 ± 1.6 | 0.09 ± 0.03 |
| 1.0% PMF$^T$ | 97.8 ± 11.9 | 0.41 ± 0.15 | 7.9 ± 1.6 | 0.07 ± 0.03 |
| 1.0% Hesp/Nar | 97.7 ± 11.9 | 0.58 ± 0.19 | 7.3 ± 1.7 | 0.08 ± 0.03 |

tions were as described above. Postcolumn split to PDA and mass ZQ detector was 10:1. MS parameters were as follows: ionization mode, ES+; scan range, 150−900 amu; scan rate, 1 scan/s; cone voltage, 20 eV.

Measurements of the metabolite levels were done similar to the method described by Nielsen et al. (*18*). Analysis of the flavonoid metabolites in hamster tissues was facilitated by the close similarities in the UV spectra of the metabolites and of Tan and Nob. These similarities permitted estimates of the levels of these compounds to be made through using conversion factors (peak areas/$\mu$g) previously determined for Tan and Nob, adjusted for each metabolite's molecular weight.

## RESULTS

**Dietary PMFs Reduce Serum Lipids and Variably Affect Hepatic Lipids.** Growth performance, serum lipid profiles, and liver lipid responses in experiments 1 and 2 are presented in **Tables 2−4**. The results show that in both studies, flavonoid supplements had no effect on body weights, food consumption, and food efficiency (**Table 2**). In experiment 1, the diet containing 1.0% Tan produced a significant reduction in serum total and VLDL + LDL cholesterol (25 and 39%, respectively) and also reduced serum triacylglycerols by 48% ($p < 0.05$) (**Table 3**). Treatment with the 1.0% Tan/Nob diet was associated

with very similar effects; significant 19 and 32% decreases in serum total and VLDL + LDL cholesterol were observed, as well as a trend for the reduction in serum triacylglycerol levels. The 0.25% Tan/Nob diet did not significantly decrease serum lipids although it tended to decrease serum total and VLDL + LDL cholesterol. The observed changes in blood lipids were not correlated with growth rate, food consumption, or food efficiency. In the liver, all three diets either induced or tended to induce increases in total, free, and esterified cholesterol and at the same time, they decreased or tended to decrease liver triacylglycerols. However, in the groups fed Tan/Nob diets, the respective increases and decreases in liver lipids did not appear to be dose-dependent (**Table 3**).

In experiment 2, the dietary treatment with 1.0% PMF$^T$ significantly reduced serum total and VLDL + LDL cholesterol by 27 and 40%, respectively, and also significantly reduced serum triacylglycerols by 44% ($p < 0.05$). These beneficial changes closely resembled those produced by the 1.0% Tan and 1.0% Tan/Nob diets in experiment 1. Treatment with 3.0% Hesp/Nar induced serum lipid responses comparable to those obtained with 1.0% Tan, Tan/Nob, and PMF$^T$ supplements, by significantly decreasing serum total and VLDL + LDL cholesterol by 28 and 38%, respectively, and also by decreasing serum triacylglycerols by 57% ($p < 0.05$). These results demonstrated that in the hamster model, dietary PMFs have greater hypolipidemic potential than the mixture of Hesp and Nar. The diet containing 1.0% PMF$^T$ did not significantly alter liver total, free, or esterified cholesterol, but it tended to reduce liver triacylglycerols (by 45%), and this effect was significant when assessed by Student's *t*-test ($p < 0.05$). In contrast, treatment with the 3.0% Hesp/Nar diet was associated with significant increases in liver total, free, and esterified cholesterol ($p < 0.05$) and with less pronounced, nonsignificant decreases in liver triacylglycerols (**Table 4**). The PMF-induced changes in liver lipids tended to be similar in both studies. However, the increases in liver total, free, and esterified cholesterol observed in experiment 2 were relatively small as compared to those in experiment 1.

**Table 3.** Serum and Liver Lipid Responses in Experiment 1 (Means ± SD)

| diet (*n*) | serum lipids (mmol/L) | | | | liver lipids (mg/g) | | | |
|---|---|---|---|---|---|---|---|---|
| | TC | VLDL + LDL C | HDL C | TG | TC | FC | CE | TG |
| control (10) | 7.15 ± 0.82 | 3.45 ± 0.51 | 3.70 ± 0.44 | 3.21 ± 1.42 | 10.1 ± 2.7 | 2.3 ± 0.2 | 7.7 ± 2.7 | 4.2 ± 1.4 |
| 0.25% Tan/Nob (5) | 6.49 ± 0.93 (−9%) | 2.79 ± 0.43 (−11%) | 3.70 ± 0.51 | 2.10 ± 1.45 (−35%) | 16.1 ± 0.9$^b$ (+59%) | 2.9 ± 0.1 (+26%) | 13.2 ± 0.9$^a$ (+71%) | 1.6 ± 1.1$^a$ (−62%) |
| 1.0% Tan/Nob (5) | 5.80 ± 0.61$^a$ (−19%) | 2.33 ± 0.26$^a$ (−32%) | 3.47 ± 0.60 | 1.97 ± 0.74 (−39%) | 16.1 ± 3.8$^b$ (+59%) | 3.4 ± 1.0$^b$ (+48%) | 12.2 ± 4.8 (+58%) | 3.1 ± 1.6 (−26%) |
| 1.0% Tan (5) | 5.37 ± 0.37$^a$ (−25%) | 2.09 ± 0.56$^a$ (−39%) | 3.28 ± 0.28 | 1.68 ± 0.61$^c$ (−48%) | 14.7 ± 3.5$^a$ (+46%) | 3.4 ± 0.5$^b$ (+48%) | 11.3 ± 3.9 (+47%) | 3.0 ± 1.7 (−29%) |

*$^a$* Significantly different from control by ANOVA + Dunnett's test, $p < 0.05$. *$^b$* Significantly different from control by ANOVA + Dunnett's test, $p < 0.01$. *$^c$* Significantly different from control by Student's *t*-test, $p < 0.01$.

**Table 4.** Serum and Liver Lipid Responses in Experiment 2 (Means ± SD)

| diet (*n*) | serum lipids (mmol/L) | | | | liver lipids (mg/g) | | | |
|---|---|---|---|---|---|---|---|---|
| | TC | VLDL + LDL C | HDL C | TG | TC | FC | CE | TG |
| control (9) | 7.33 ± 0.65 | 3.57 ± 0.35 | 3.75 ± 0.44 | 3.47 ± 1.24 | 9.7 ± 2.8 | 2.6 ± 0.4 | 7.2 ± 2.9 | 4.4 ± 1.2 |
| 1.0% PMF$^T$ (5) | 5.32 ± 0.85$^b$ (−27%) | 2.13 ± 0.59$^b$ (−40%) | 3.20 ± 0.30 (−15%) | 1.94 ± 0.75$^a$ (−44%) | 12.1 ± 4.5 (+25%) | 3.4 ± 1.5 (+31%) | 7.7 ± 4.7 | 2.4 ± 1.9$^c$ (−45%) |
| 3.0% Hesp/Nar (5) | 5.29 ± 0.72$^b$ (−28%) | 2.20 ± 0.57$^b$ (−38%) | 3.09 ± 0.29 (−18%) | 1.49 ± 1.10$^b$ (−57%) | 17.0 ± 4.6$^b$ (+75%) | 4.6 ± 0.5$^b$ (+77%) | 12.4 ± 4.2$^a$ (+72%) | 3.4 ± 3.0 (−23%) |

*$^a$* Significantly different from control by ANOVA + Dunnett's test, $p < 0.05$. *$^b$* Significantly different from control by ANOVA + Dunnett's test, $p < 0.01$. *$^c$* Significantly different from control by Student's *t*-test, $p < 0.05$.
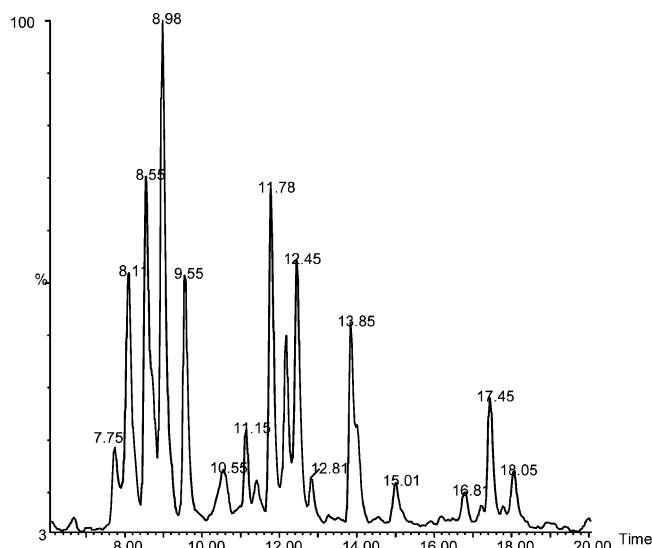
**Figure 2.** HPLC profile (330 nm) of Tan metabolites in hamster serum solids. The molecular ion $(M + H)^{+1}$ masses and the main flavone fragment ions of each of the main metabolites are listed in **Table 5**.

**Table 5.** Tan Metabolite Concentrations in Blood Serum Solids from Hamsters Fed a Diet Containing 1% Tan[a]

| peak elution time (min) | molecular weight/ aglycone (amu) | flavone derivative | concn ($\mu$g/g) |
|---|---|---|---|
| 8.11 | 521/345 | dihydroxytrimethoxy | 15.0 ± 1.6 |
| 8.55 | 521/345 | dihydroxytrimethoxy | 18.1 ± 3.7 |
| 8.98 | 521/345 | dihydroxytrimethoxy | 25.4 ± 4.6 |
| 9.55 | 521/345 | dihydroxytrimethoxy | 15.3 ± 5.8 |
| 11.78 | 535/359 | monohydroxytetramethoxy | 10.6 ± 5.4 |
| 12.10 | 535/359 | monohydroxytetramethoxy | 4.9 ± 2.5 |
| 12.45 | 345 | dihydroxytrimethoxy | 7.9 ± 1.8 |
| 13.85 | 535/359 | monohydroxytetramethoxy | 11.6 ± 5.6 |
| 17.45 | 359 | monohydroxytetramethoxy | 2.0 ± 0.8 |

[a] Blood serum was freeze-dried, and the dried powder was extracted with 2.0 mL of dimethyl sulfoxide/methanol (1/1). Values are averages from five animals. Peaks are identified according to their retention times. Molecular weights $(M + 1)^{+}$ and aglycone fragment ions $(M + 1)^{+}$ for each peak are listed.

**Identification and Quantitation of PMF Metabolites in Serum, Liver, and Urine.** Blood serum, liver tissues, and urine were sampled from the hamsters at the completion of the Tan and Tan/Nob feeding trials. The metabolites in these tissues were analyzed by HPLC with PDA detection and further characterized by HPLC-MS, where the $(M + H)^{+1}$ molecular and fragment ions provided structural information about these compounds. A representative HPLC chromatogram of the metabolites in blood serum of hamsters fed the 1% TAN diet is shown in **Figure 2**. Nearly all of the peaks in this chromatogram showed UV spectra similar to that of Tan (data not shown). The molecular weights of the main metabolites in this chromatogram are listed in **Table 5**, along with the flavone aglycone metabolite ions and the estimated concentrations of these metabolites in the blood serum solids. Most metabolites in the serum of hamsters fed the 1% Tan diet occurred as glucuronides of 345 and 359 amu species. The early eluting peaks had molecular weights $(M + H)^{+1}$ of 521 amu and major fragment ions at 345 amu, where these latter ions were increasingly evident at higher collisional energies. These data indicate the occurrence of glucuronides (176 amu) of dihydroxytrimethoxyflavones (345 amu). In contrast, most of the later eluting metabolites showed $(M + H)^{+1}$ ions at 535 amu, consistent with the occurrence of

glucuronides of monohydroxytetramethoxyflavones. Trace levels of the unconjugated 345 and 359 amu aglycones were also detected (**Table 5**), along with trace levels of unchanged Tan. No sulfated Tan metabolites were detected in these samples, as indicated by the absence of 80 amu fragment losses in any of the metabolites shown in **Figure 2**. The quantitative data demonstrated that Tan metabolites in serum solids consisted largely of glucuronides (91%) and that 74% of all metabolites present in the serum were either free or conjugated derivatives of dihydroxytrimethoxyflavones. The total concentration of all metabolites in serum solids was around 110 $\mu$g/g, an equivalent of 21 $\mu$M intact Tan in the fresh serum. No relationship was found between total concentrations of PMF metabolites in serum and blood lipids.

A representative HPLC chromatogram of liver extracts obtained from hamsters fed the diet of 1% Tan is shown in **Figure 3**. Also shown is the enhanced detection by mass spectral SIM of the metabolites at 535, 359, 345, and 521 amu. While SIM provided detection of more than 12 Tan metabolites, only four were prominent in the chromatograms monitored at 330 nm. The mass spectra of the four main metabolites indicated the occurrence of a dihydroxytrimethoxyflavone glucuronide at 9.05 min and were consistent with the occurrence of three dihydroxytrimethoxyflavone or monohydroxytetramethoxyflavone aglycones at 12.41, 13.15, and 18.58 min, respectively. Liver extracts contained only trace amounts of monohydroxytetramethoxyflavone glucuronides (535 amu) although these compounds were abundant in the blood serum. Estimated concentrations of the principal Tan metabolites in the liver extracts of hamsters fed 1% Tan diet are listed in **Table 6**. Only 35−59% of the metabolites were glucuronides (**Table 7**), consisting mainly of the dihydroxytrimethoxyflavone metabolite at 9.05 min. Among the unconjugated aglycones, 79−91% were identified as dihydroxytrimethoxyflavones and 10−21% were monohydroxytetramethoxyflavones. The total concentrations of all hepatic Tan metabolites found in the liver solids from hamsters fed 1.0% Tan or 1.0% Tan/Nob diets were within the range of 25−106 $\mu$g/g. This corresponded to 16−67 $\mu$M in the fresh tissue. The PMF metabolite concentrations in the liver tissue were not correlated with blood lipid results.
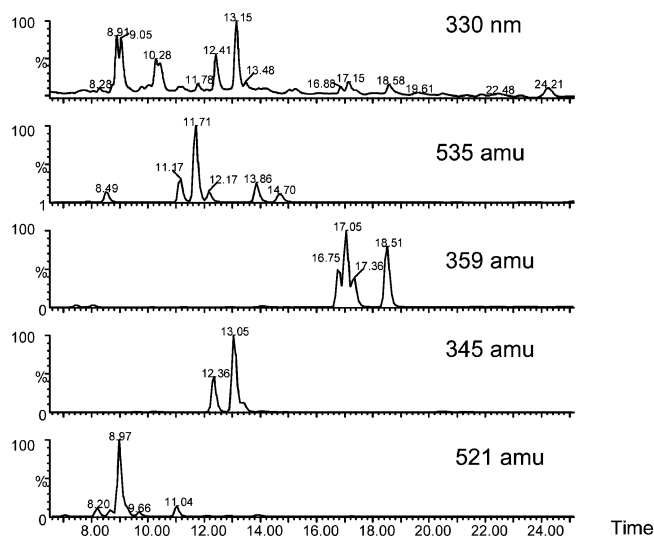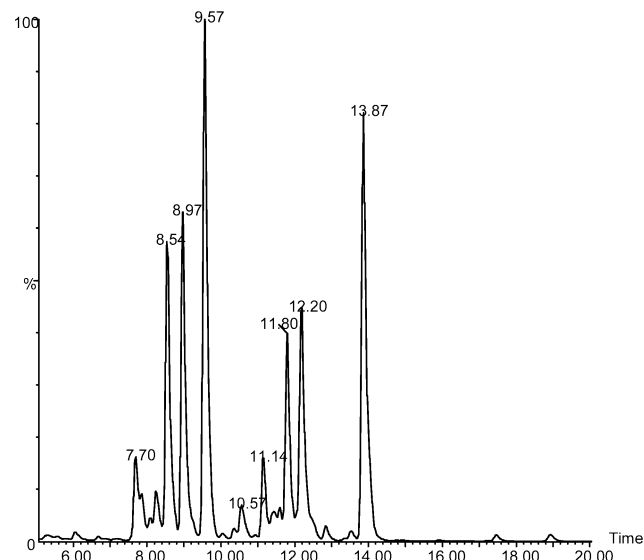


**Figure 3.** HPLC-MS of Tan metabolites in hamster liver extracts. The upper part represents the HPLC profile of the extract at 330 nm. Below are enhanced SIM profiles of 535, 359, 345, and 521 amu. The molecular ion $(M + H)^{+1}$ masses and the flavone fragment ions of each of the main metabolites are listed in **Table 6**.

Citrus Polymethoxylated Flavones in Hamsters

*J. Agric. Food Chem.,* Vol. 52, No. 10, 2004 **2883**

**Table 6.** Tan Metabolite Concentrations in Liver Extracts from Hamsters Fed a Diet Containing 1% Tan[a]

| peak elution time (min) | molecular weight/ aglycone (amu) | metabolite concn ($\mu$g/g) | concn range ($\mu$g/g) |
|---|---|---|---|
| 8.97 | 521 | trace | trace |
| 9.05 | 521/345 | 31.8 ± 19.0 | 14.3–62.9 |
| 11.17 | 535/359 | trace | trace |
| 11.71 | 535/359 | trace | trace |
| 12.41 | 345 | 11.0 ± 6.6 | 4.2–19.3 |
| 13.15 | 345 | 16.0 ± 10.4 | 5.1–14.9 |
| 16.75 | 359 | trace | trace |
| 17.05 | 359 | trace | trace |
| 17.36 | 359 | trace | trace |
| 18.51 | 359 | 4.7 ± 2.8 | 1.7–8.9 |
| 25.9 | 373 | trace | trace |
| total | | 63.4 ± 31.8 | 25.3–106.0 |

[a] Values are averages from five animals. Peaks are identified as in **Table 5**.

**Table 7.** Percent Distribution of Tan Molecular Species in Liver Samples from Hamsters Fed 1% Tan Diet

| | percent content | range |
|---|---|---|
| glucuronides (% of total) | 50 ± 12 | 35–59 |
| free aglycones (% of total) | 50 ± 12 | 38–65 |
| dihydroxytrimethylflavones (% of free aglycones) | 85 ± 4 | 79–91 |
| monohydroxytetramethylflavones (% of free aglycones) | 15 ± 4 | 10–21 |



**Figure 4.** HPLC profile (330 nm) of Tan metabolites in hamster urine. The molecular ions (M + H)$^{+1}$ and the flavone fragment ions of each of the main metabolites are listed in **Table 8**.

**Table 8.** Tan and Nob Metabolites in Hamster Urine[a]

| peak elution time (min) | molecular weight/ aglycone (amu) | diets | | |
|---|---|---|---|---|
| | | 0.25% Tan/Nob | 1% Tan/Nob | 1% Tan |
| 8.54 | 521/345 | 406 ± 133 | 2328 ± 611 | 1333 ± 225 |
| 8.97 | 521/345 | 336 ± 160 | 2372 ± 592 | 1422 ± 386 |
| 9.57 | 521/345 | 98 ± 17 | 646 ± 81 | 1083 ± 298 |
| 11.14 | 535/359 | 177 ± 54 | 967 ± 240 | 264 ± 65 |
| 11.80 | 535/359 | 200 ± 47 | 400 ± 71 | 496 ± 181 |
| 12.20 | 535/359 | 155 ± 52 | 1050 ± 234 | 726 ± 146 |
| 12.84 | 565/389 | 66 ± 35 | 1100 ± 188 | none |
| 13.57 | 345 | 8.7 ± 3.8 | 32.2 ± 6.1 | 20.1 ± 8.2 |
| 13.87 | 535/359 | 304 ± 95 | 2045 ± 563 | 763 ± 365 |
| 17.47 | 359 | 3.6 ± 1.2 | 15.3 ± 2.1 | 10.4 ± 3.4 |
| 18.94 | 359 | 11.1 ± 5.3 | 29.6 ± 7.5 | 10.9 ± 4.5 |

[a] Values are averages of five animals per trial. Urine was collected on the last day of the feeding trials and freeze-dried. Weighed fractions of the freeze-dried urine samples were dissolved in 3.0 mL of dimethyl sulfoxide, filtered, and analyzed by HPLC-MS. The peak with a molecular weight of 565/389 has been identified as desmethylnobiletin. Other peaks are identified as in **Table 5**.
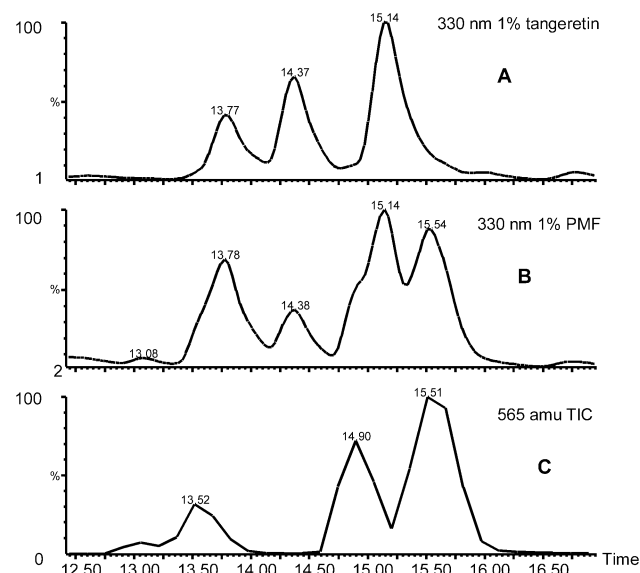


**Figure 5.** (A,B) Chromatograms of the 1% Tan and 1% Tan/Nob diet metabolite profiles, respectively, monitored at 330 nm. (C) Derived total ion current at 565 amu, illustrating the additional peak at 15.54 min, and the early-eluting shoulders on the peaks at 13.77 and 15.14 min.

13.77 and 15.14 min are due to the additional metabolites with the 389/565 mass ions. The fragment ion at 389 amu is consistent with a monohydroxypentamethoxyflavone, where a methoxyl group on Nob was converted to a hydroxyl substituent, suggesting that the molecular ion at 565 amu can be tentatively assigned to a glucuronide of a monohydroxypentamethoxyflavone (desmethylnobiletin) metabolite. The UV spectra of the 565/389 amu metabolites in **Figure 5** closely matched the UV spectrum of Nob, while they differed significantly from the spectra of the metabolites putatively formed from Tan (data not shown).

The SIM-enhanced HPLC profiles of urine from hamsters fed Tan and Tan/Nob diets showed trace levels of unchanged Tan and Nob. SIM further showed the occurrence of trace levels of metabolites with mass ions at 375 and 551, corresponding to possible dihydroxytetramethoxyflavones, which were previously found at much larger concentrations in the urine obtained of rats fed Tan-enriched diet (*18*).

As demonstrated in **Table 8**, almost all PMF species from the urine (99%) were glucuronides. In the samples from Tan-

The metabolite profile of the urine obtained from hamsters fed the 1% Tan diet is shown in **Figure 4**. **Table 8** lists the molecular weights of the main metabolites along with their estimated concentrations in urine obtained from animals fed the 1% Tan, 0.25% Tan/Nob, and 1% Tan/Nob diets. The metabolite profiles of all three treatment groups (**Figure 4**) were similar to the profile of the blood serum (**Figure 2**). The Tan/Nob diet groups contained additional (M + H)$^{+1}$ peaks at 565 amu and fragment ions at 389 amu (**Figure 5A**−**C**). **Figure 5A**,**B** shows the contrasting UV chromatograms of the 1% Tan and 1% Tan/ Nob diet metabolite profiles. **Figure 5C** shows the extracted total ion current at 565 amu, illustrating that the additional peak at 15.54 min and the early eluting shoulders on the peaks at

fed animals, 63% of the total concentrations of these conjugates occurred as derivatives of dihydroxytrimethoxyflavones and the remaining 37% occurred as derivatives of monohydroxytetramethoxyflavones. The urine extracts from groups fed Tan/Nob diets contained a slightly lower proportion of the dihydroxytrimethoxy conjugates (48−49%), higher levels of the monohydroxytetramethoxy derivatives (41−48%), and additionally, 4−10% of the conjugated Nob product, desmethylnobiletin. The total concentrations of PMF metabolites in the fresh urine from hamsters fed 1.0% Tan or 1.0% Tan/Nob diets were in the range from 11 to 21 mM, and correspondingly lower levels of metabolites were detected in the urine from animals fed the 0.25% Tan/Nob diet. Concentrations of PMF metabolites found in urine samples from hamsters fed PMF-supplemented diets were not correlated with blood lipid responses.

## DISCUSSION

Our results demonstrate for the first time that in hamsters with casein-induced hypercholesterolemia, diets containing 1% PMFs (Tan, Tan/Nob, or PMF$^T$) significantly reduced serum total and VLDL + LDL cholesterol and either reduced or tended to reduce serum triacylglycerols without altering HDL cholesterol levels and without causing toxic effects. The trend for decline in serum total and VLDL + LDL cholesterol was also observed in the animals fed 0.25% PMFs. The decreases in serum cholesterol and triacylglycerol concentrations observed in hamsters fed the 1% PMF$^T$ diet were comparable to those obtained by feeding three times higher combined levels of Hesp and Nar, suggesting that the PMFs had greater lipid lowering potential than Hesp and Nar. This was in agreement with our previous report showing a greater hypolipidemic potential of Tan and Nob vs hesperetin and naringenin in human liver cell line HepG2 (*17*) and with data suggesting a relatively weak hypolipidemic action of Hesp and Nar in vivo (*15*). Also, a preliminary study of ours showed that in hamsters with casein-induced hypercholesterolemia, a supplementation with 2% Hesp or 2% Nar had little effect on blood cholesterol (unpublished). The beneficial changes in serum lipids produced by dietary supplementation with PMFs were associated with variable and inconsistent increases in liver cholesterol and also with more consistent decreases in liver triacylglycerols. The elevated hepatic levels of total, free, and esterified cholesterol observed in some but not all experimental groups remain unexplained since earlier studies in rats and mice fed Hesp and Nar showed a significant reduction in liver concentrations of cholesterol and triacylglycerols (*13*, *14*).

The substantial hypolipidemic potential of PMFs observed both in hamsters and in human hepatoma HepG2 cells suggests that PMFs or their metabolites modulate lipoprotein and lipid metabolism directly in the liver. However, until now, the Tan derivatives, which might be responsible for hypolipidemic effects in the liver tissue, have not been identified. Thus, in the present study, we analyzed by HPLC-MS the metabolites of PMFs formed in vivo in the serum, liver, and urine of hamsters fed Tan- or Tan and Nob-enriched diets.

Serum samples from animals given 1% Tan diet contained seven major glucuronic acid conjugates and only two aglycones. Glucuronides were present in serum solids at much higher concentrations than aglycones, accounting for more than 90% of all serum metabolites. The almost complete glucuronidation of circulating Tan products was anticipated since the formation of glucuronide conjugates in the gut mucosa was commonly observed for other flavonoids and xenobiotic compounds (*26*). The presence of small amounts of unconjugated Tan derivatives

and the absence of sulfate conjugates in the blood serum were also in agreement with earlier data. Low concentrations of free flavonoid forms have been suggested to enter the circulation due to saturation of the glucuronidation pathway and/or due to enterohepatic cycling and hydrolysis of conjugates by colonic bacteria (*26−28*). Likewise, only a limited sulfate formation was observed in rats administered a flavonol, quercetin, and this was postulated to be due to the low activity of intestinal sulfotransferase and/or the high concentration of quercetin in the gut (*29*).

The mass spectra of the conjugated and unconjugated Tan products found in the serum, liver, and urine from hamsters fed 1% Tan diet suggested that the metabolites occurred primarily as dihydroxytrimethoxyflavones and monohydroxytetramethoxyflavones. These two categories of metabolites were also detected in the urine and feces of Tan-fed rats but in this species, unlike in hamsters, monohydroxytetramethoxyflavones were predominant (*18*). Furthermore, the urine from Tan-fed rats also contained substantial amounts of another metabolite, dihydroxytetramethoxyflavone, a compound that was present only at trace levels in the hamster urine. This discrepancy between hamster and rat Tan metabolism is intriguing since significant amounts of dihydroxytetramethoxyflavone were also found in liver microsomal fractions exposed to Tan in vitro (*20*, *21*).

The profiles of Tan metabolites in the liver extracts from hamsters fed 1% Tan diet were considerably different from those in the serum. Tan metabolites in the liver included four major products, and the mass spectra of these compounds indicated that only one of them occurred as a glucuronide. Also, liver samples contained lower proportions of glucuronides than serum specimen (35−59% vs more than 90% in the serum) and virtually all hepatic metabolites of Tan were identified as conjugates of dihydroxytrimethoxyflavones. The unconjugated Tan derivatives, which were present in liver samples at relatively high levels, consisted of 80−90% dihydroxytrimethoxyflavones and 10−20% monohydroxytetramethoxyflavones whereas in the serum, the proportions of dihydroxy to monohydroxy derivatives were 74 and 26%, respectively.

The total concentrations of Tan metabolic products in the liver tissue from hamsters fed 1% Tan diet were in the range equivalent to 16−67 $\mu$M free Tan and thus corresponded to the concentrations of parent PMFs, Tan and Nob, which produced significant hypolipidemic responses in human hepatoma HepG2 cells in our previous study in vitro (*17*). Because intact hepatocytes have been shown to metabolize flavonoids, mostly to glucuronides (*28*, *30*, *31*), this suggested that the elevated levels of PMF metabolites in the liver could be directly responsible for the PMF-induced changes in the hepatic lipoprotein and lipid metabolism in vivo.

The PMF biotransformation products found in the urine of hamsters fed PMF-enriched diets were generally similar to the products detected in the serum, except the total concentrations of urinary PMF metabolites were substantially higher and 99% of the compounds in the urine were glucuronidated. However, the urinary PMF profiles in hamsters fed the Tan-enriched diet differed from the profiles obtained for Tan-fed rats with respect to the number and proportions of major isomers (as discussed earlier) and also with respect to the degree of glucuronidation. While in the hamsters, urinary PMFs were almost completely glucuronidated, and only partial conjugation with glucuronate and/or sulfate occurred in rats (*18*).

Our results suggest that there were at least three metabolites of Nob in the urine of hamsters fed the 1% Tan/Nob diet.

Citrus Polymethoxylated Flavones in Hamsters

*J. Agric. Food Chem.,* Vol. 52, No. 10, 2004 **2885**

Although the structures of the desmethylnobiletin metabolites tentatively identified by MS in the hamster urine have not been elucidated, one isomer of this compound, 3′-desmethylnobiletin, has also been detected in substantial quantities in the serum and urine from rats given a single dose of Nob (*19*) and in vitro, following the incubation of Nob with human intestinal Caco-2 cells or with rat liver microsomes (*22*).

In summary, our results demonstrate that dietary Tan and other PMFs induce substantial hypolipidemic responses in hamsters with experimental hypercholesterolemia, and the lipid lowering potential of PMFs is greater than that of a mixture containing Hesp and Nar. The beneficial effects of PMFs were associated with their extensive absorption and metabolism, as confirmed by the substantial presence of several PMF biotransformation products in the serum, liver, and urine. The combined concentrations of Tan metabolites found in hamster liver extracts appear to be sufficient to modulate lipoprotein and lipid metabolism in this organ, as confirmed by our earlier studies in human hepatoma HepG2 cells exposed to closely corresponding concentrations of intact Tan. As the mechanisms by which Tan affects HepG2 cell lipoprotein and lipid metabolism have recently been investigated by our group (*32*), subsequent studies should be done to examine the potential therapeutic applications of PMFs in the treatment of hypercholesterolemia and hypertriglyceridemia.

## ABBREVIATIONS USED

PMFs, polymethoxylated flavones; VLDL, very low-density lipoproteins; LDL, low-density lipoproteins; Tan, tangeretin; Nob, nobiletin; PMF$^T$, PMF from tangerines; Hesp, hesperidin; Nar, naringin; HDL, high-density lipoproteins; HPLC, high-performance liquid chromatography; PDA, photodiode array; MS, mass spectrometry; amu, atomic mass units; SIM, selective ion monitoring.

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**2886** *J. Agric. Food Chem.,* Vol. 52, No. 10, 2004

Kurowska and Manthey

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